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16. ABSTRACT

The ability of seven methylating agents to form 7-methylguanine and O^6-methyl - guanine was compared to their ability to initiate carcinogenesis as measured by the initiation of GGTase-positive foci. The seven methylating agents studied were diazald, DMH, DMN, DMS, MMS, MNNG, and MNU. The DNA methylation and initiation of GGTase-positive foci was determined in partial hepatectomized rats. The formation of foci was promoted by 500 ppm sodium phenobarbital in the drinking water. While six of the seven compounds (DMH, DMN, DMS, MMS, MNNG and MNU) producted 7-methylquanine, only the four compounds (DMH, DMN, MNNG and MNU) that produced O^6-methylguanine initiated GGTase-positive foci. The extent of O^6-methylguanine produced by the methylating agents did not correspond with their potency to initiate GGTasepositive foci. Therefore, the initiation of GGTase-positive foci required the formation of O^6-methylguanine. However, some sequential event altered the quantitative relationship of O^6-methylguanine formation to the incidence of GGTase-positive foci.

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ROLE OF O^6-METHYLATION IN THE INITIATION OF GGTase-POSITIVE FOCI

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SUMMARY

The ability of seven methylating agents to form 7-methylguanine and O^6-methylguanine was compared to their ability to initiate carcinogenesis as measured by the initiation of GGTase-positive foci. The seven methylating agents studied were methyl-*N*-nitroso-*p*-toluenesulfonamide (diazald), dimethylhydrazine (DMH), dimethylnitrosamine (DMN), dimethylsulfate (DMS), methyl methanesulfonate (MMS), methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) and methyl-*N*-nitrosourea (MNU). The DNA methylation and initiation of GGTase-positive foci was determined in partial hepatectomized rats. The formation of foci was promoted by 500 ppm sodium phenobarbital in the drinking water. While six of the seven compounds (DMH, DMN, DMS, MMS, MNNG and MNU) produced 7-methylguanine, only the four compounds (DMH, DMN, MNNG and MNU) that produced O^6-methylguanine initiated GGTase-positive foci. The extent of O^6-methylguanine produced by the methylating agents did not correspond with their potency to initiate GGTase-positive foci. Therefore, the initiation of GGTase-positive foci required the formation of O^6-methylguanine. However, some sequential event altered the quantitative relationship of O^6-methylguanine formation to the incidence of GGTase-positive foci.

Key words: Methylation — Initiation — Carcinogenesis — Liver — Rat — DNA binding

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Abbreviations: Diazald, methyl-*N*-nitroso-*p*-toluenesulfonamide; DMH, dimethylhydrazine; DMN, dimethylnitrosamine; DMS, dimethylsulfate; DMSO, dimethylsulfoxide; MMS, methyl methanesulfonate, MNNG, methyl-*N*-nitro-*N*-nitrosoguanidine; MNU, methyl-*N*-nitrosourea.

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INTRODUCTION

Chemical carcinogenesis is believed to be a multi-step progression starting from an initiated cell and progressing through the steps of cellular transformation, preneoplastic lesions (foci), benign tumors and finally cancer [1—3]. Initiation appears to commence with the binding of the carcinogen to DNA [4—6]. Fixation of the alteration during DNA replication and subsequent expression of the alteration results in a focus of altered hepatocytes. Methylating carcinogens either directly or after metabolic activation alkylate DNA especially at the N-7 or O^6-positions of guanine [5—7]. The extent of formation of the methylation product in DNA responsible for the induction of cancer should correspond to the extent of initiation.

Cellular replication of the genetically altered and initiated cell results in the appearance of a focus of cells expressing an altered phenotype [1—3, 8—10]. GGTase-positive foci are one type of altered-foci which are proposed to be the earliest recognizable evidence for initiation [1—3]. In the present study, we report the relationship of the formation of alkylation products in DNA after exposure to methylating carcinogens to the incidence of GGTase-positive foci in order to test the relationship of the various alkylation products to the initiation of cancer.

MATERIALS AND METHODS

Animals

Male Sprague—Dawley rats (Charles River Co., Portage, MI) weighing 200—225 g were used in these studies. The animals were maintained in accordance with the standards set forth in 'Guide for the Care and Use of Laboratory Animals', Institute of Laboratory Animal Resources, National Research Council. They received Purina rodent chow (Ralston Purina Company, St. Louis, MO) and distilled water ad libitum.

Chemicals

Methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald), DMH, DMN, DMS, MMS and MNNG were purchased at the highest purity available from Aldrich (Milwaukee, WI); MNU from ICN Pharmaceuticals, Inc. (Plainview, NY); dimethylsulfoxide (DMSO) (glass distilled) frrom Burdirk and Jackson Laboratories, Inc., (Muskegon, MI); sodium phenobarbital from J.T. Baker (Phillipsburg, NJ); *N*-γ-L-glutamyl-4-methoxy-2-naphthylamide from Bachem (Torrence, CA); 1-methyladenine, 3-methyladenine, 7-methyladenine, 3-methylguanine and 7-methylguanine from P-L Biochemicals, Inc. (Milwaukee, WI). O^6-Methylguanine was synthesized by the procedure of Balsiger and Montgomery [11] and identified by GC-MS.

Radiolabelled chemicals

[^{14}C]MMS (59 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL) and [*methyl*-^{14}C]DMS (18.5 mCi/mmol), *N,N'*-

[*methyl*-^{14}C]DMH dihydrochloride (7.7 mCi/mmol) [*methyl*-^{14}C]MNNG (15.0 mCi/mmol), *N*-[*methyl*-^{14}C]diazald (7.8 mCi/mmol), *N*-[*methyl*-^{3}H]-MNU (1.6 Ci/mmol) and *N*-[*methyl*-^{14}C]nitrosodimethylamine (50.9 mCi/mmol) from New England Nuclear Corporation (Boston, MA).

DNA METHYLATION

The rats received a two thirds partial hepatectomy while under light ether anesthesia. Twenty-four hours later the radiolabeled methylating agent was administered to the rats. The animals were sacrificed 2.5 h later by cervical dislocation and the DNA isolated by a modification of the procedure described by Kirby [12]. The liver was homogenized in a lysing medium containing 1% sodium triisopropylnaphthalene sulfonate, 6% isobutanol, 6% sodium-para-animosalicylate, 1% sodium chloride and 0.01% sodium deoxycholate and incubated with proteinase K for 1 h. After extraction with Kirby's phenol solution, the DNA was precipitated with 2-ethoxyethanol. The DNA was then sequentially treated with RNase, amylase and Pronase followed by a second phenol extraction. After 2-ethoxyethanol precipitation, the DNA was dissolved in water. Contamination by protein was less than 2% as determined by the procedure of Lowry et al. [13]. For determination of radioactivity, an aliquot of the DNA was hydrolyzed in 0.1 N HCl at 70°C for 30 min, dissolved in ACS (Amersham Corp., Arlington Heights, IL), and counted in a Beckman 9000 liquid scintillation counter (Beckman Instrument Co., Palo Alto, CA). DNA concentration was determined by the procedure of Kissane and Robins [14].

A neutral hydrolysis was accomplished by dissolving the DNA in 0.002 M sodium phosphate buffer (pH 6.9) and heating for 30 min in a boiling water bath. The apurinated DNA was precipitated by the addition of 1/20 vol. of 2 N HCl. The precipitate was collected by centrifugation and washed with cold 0.1 N HCl. The supernatants were combined and after the addition of marker compounds (3- and 7-methylated adenine and guanine) were chromatographed on high pressure liquid chromatography (HPLC). The precipitate was then suspended in 0.1 N HCl and heated at 70°C for 30 min. The solution was filtered through 0.45 μm filters and after the addition of O^6-methylguanine and 1-methyladenine, chromatographed on HPLC.

The DNA degradation products were separated on a HPLC-SP8000 (spectra Physics, Santa Clara, CA) equipped with a Magnum 9 Partisil-10 strong cation exchange column (9.4 × 250 mm; Whatman Inc., Boston, MA). The column was eluted with a Spectra Physics #5 non-linear gradient starting with 94% 0.025 M ammonium phosphate (pH 4.0—6% methanol) and ending with 94% 0.25 M ammonium phosphate (pH 4.0—6% methanol). The gradient took 25 min and the flow rate was 4 ml/min. Absorbance was monitored at 260 nm with a ISCO Model spectrophotometer (Instrumentation Specialities Co., Lincoln, NE). Eluted fractions (2-ml) were collected into scintillation vials and after the addition of 15 ml ACS (Amersham Corp.,

Arlington Heights, IL) the radioactivity was measured in a Beckman 9000 liquid scintillation counter (Beckman Instrument Co., Palo Alto, CA).

Initiation of GGTase-positive foci

Two protocols of the initiation assay were employed [3]. In protocol 1, the rats received a two-thirds partial hepatectomy while under light ether anesthesia and 20—22 h later were administered intragastrically the methylating agent. One week later the rats started to receive 500 ppm sodium phenobarbital in their drinking water. The rats received phenobarbital for 7 weeks and were sacrificed 1 week after the cessation of the phenobarbital treatment. The liver was excised and blocks frozen in O.C.T. compound. In protocol 2, intact rats received the methylating agent and 1 week later they started to receive 500 ppm sodium phenobarbital. Two weeks after the methylating agent was given, a two-thirds partial hepatectomy was performed. At this time the phenobarbital concentration was decreased to 100 ppm. One week after surgery, the concentration of phenobarbital was increased to 500 ppm until 1 week prior to sacrifice. The rats were sacrificed 9 weeks after the methylating agent was given. The liver was excised and blocks frozen in O.C.T. compound. Cryostatic sections (6 μm) were obtained at −25°C and stained for GGTase activity by the procedure of Rutenburg [15]. The sections were counterstained with hematoxylin. At least 2 cm^2 of sections from each animal were scanned for GGTase-positive foci containing at least nine nuclei.

RESULTS

Methylation of DNA

A representative HPLC elution profile depicting the separation of 3- and 7-methyladenine and 3- and 7-methylguanine present in a neutral hydrolysate is shown in Fig. 1a. The levels of 7-methylguanine and 3-methyladenine formed in vivo were readily obtained from the elution profile. Though 3-methylguanine and 7-methyladenine were separated, their levels were below the sensitivity required for quantitation. The elution profile shown in Fig. 1b depicts the separation by HPLC of the O^6-methylguanine and 1-methyladenine that were present in an acid hydrolysate. Only O^6-methylguanine was present in a sufficient amount for quantitation.

The levels of 7-methylguanine, O^6-methylguanine and 3-methyladenine formed by the seven methylating agents in regenerating rat liver are presented in Table I. The seven methylating agents were administered at the same dose and in the same solvent used to determine their ability to initiate GGTase-positive foci. Under the conditions used the rank order of the formation of 7-methylguanine was MNNG = DMN > MNU > MMS > DMH = DMS > Diazald, O^6-methylguanine was DMN > MNNG > MNU > DMH > Diazald = DMS = MMS and 3-methyladenine was DMN > MNNG > MMS > MNU > DMH = DMS > Diazald.

The ratio of the formation of O^6-methylguanine to 7-methylguanine is

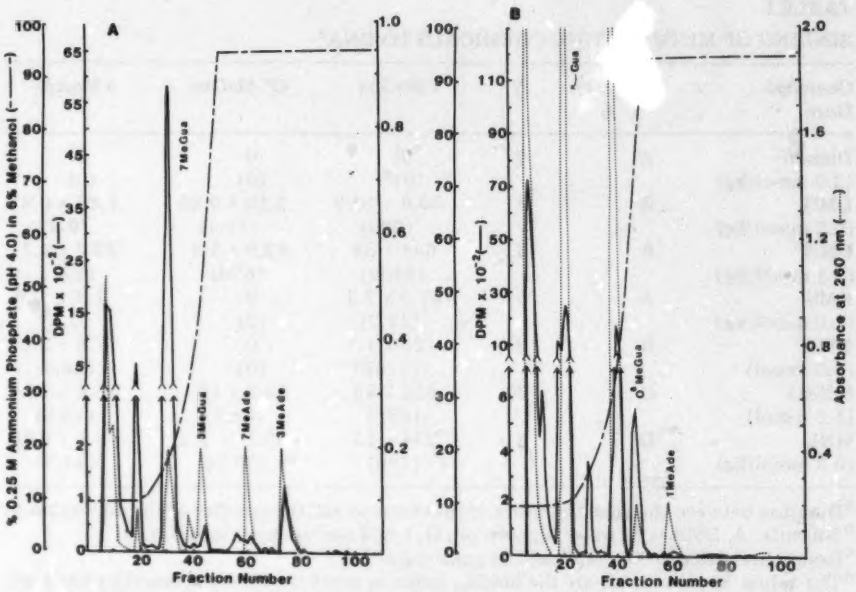

Fig. 1. HPLC elution profiles of methylated bases in liver DNA. Rats were treated with [^{14}C]DMN and the liver DNA isolated. A: Neutral hydrolysis. The isolated DNA was hydrolyzed at pH 6.9 for 30 min in a boiling water bath. The apurinated DNA was removed by precipitation with 0.1 N HCl and marker compounds, 3- and 7-methylated adenine and quanine, added to the hydrolysate. B: Acid hydrolysis. The apurinated DNA was hydrolyzed in 0.1 N HCl at 70°C for 30 min. The solution was filtered and the marker compounds, O^6-methylguanine and 1-methyladenine, added. The neutral and acid hydrolysates of the DNA were applied separately to a Magnum 9 Partisil-10 strong cation exchange column (9.4 × 250 mm). The column was eluted with a Spectra Physics #5 non-linear gradient starting with 94% 0.025 M ammonium phosphate (pH 4.0—6% methanol) and ending with 94% 0.25 M ammonium phosphate (pH 0.0—6% methanol).

dependent on the mechanism by which the methylating agent reacts with the nucleophilic sites in the DNA. S_N1 alklating agents compared to S_N2 agents bound to a greater extent the O^6 position of guanine relative to the amount bound at the N-7 position (Table II). The S_N2 alkylating agents had a ratio of O^6/7-methylguanine of zero and the S_N1 agents had a ratio that approached 0.1. The ratio of 3-methyladenine/7-methylguanine for the six methylating agents that bound DNA varied between 0.02 and 0.06, and did not correspond to the mechanism by which the agent bound nucleophilic sites.

Initiation of GGTase-positive foci

The ability of the methylating agents to initiate GGTase-positive foci was determined in rats that were treated with non-radiolabelled chemical identical to the treatment with the radiolabelled chemical. Thus, the rats were ad-

TABLE I

BINDING OF METHYLATING CHEMICALS TO DNA[a]

Chemical Dose	Solvent[b]	*N*	7-MeGua	O^6-MeGua	3-MeAde
Diazald (2.0 mmol/kg)	A	2	0[c] (0)[d]	0 (0)	0 (0)
DMH (0.2 mmol/kg)	B	3	39.8 ± 10.9 (200)	2.15 ± 0.85 (11.4)	1.85 ± 0.87 (9.3)
DMN (0.1 mmol/kg)	B	6	648 ± 88 (6480)	62.9 ± 2.4 (629)	23.7 ± 4.7 (237)
DMS (1.0 mmol/kg)	A	5	37.3 ± 7.3 (37.3)	0 (0)	1.3 ± 0.9 (1.3)
MMS (0.3 mmol)	B	2	126 ± 4.7 (420)	0 (0)	7.6 ± 2.2 (25.3)
MNNG (1.0 mmol)	C	3	652 ± 86 (652)	54.3 ± 10 (54.3)	15.6 ± 11.3 (15.6)
MNU (0.3 mmol/kg)	D	3	234 ± 13 (780)	23.1 ± 2.2 (77.0)	3.5 ± 0.18 (11.7)

[a] Duration between the administration of the chemical and the sacrifice of the rat was 2.5 h.
[b] Solvents: A, DMSO: B, water; C, corn oil; D, 1 mM sodium acetate (pH 5.6).
[c] Results are means ± S.E. expressed as pmol/mg DNA.
[d] The values in parenthesis are the binding index as pmol/mg DNA per mmol/kg body wt.

ministered the methylating agent 20—24 h after a two-thirds partial hepatectomy. The partial hepatectomy was performed before administering the methylating agent because it had previously been shown to increase their response in the liver foci assay [8,9]. We also determined their initiating activity in a protocol that included that partial hepatectomy at 2 weeks after the administration of the methylating agents. This second protocol was used in order to determine the extent to which the methylating agents were able to initiate hepatocarcinogenesis without prior partial hepatectomy. DMH and DMN initiated an approximately equal number of GGTase-positive foci which were the highest incidence produced by the methylating agents (Table III). The dose of DMN given after the partial hepatectomy was

TABLE II

RELATIONSHIP OF ALKYLATION PRODUCTS TO MECHANISM OF ALKYLATION

Chemical	Mechanism	Ratio O^6/N-7 MeGua	Ratio 3-MeAde/ N-7 MeGua
DMS	S_N2	0	0.02
MMS	S_N2	0	0.06
DMH	S_N1/S_N2	0.054	0.04
MNNG	S_N1	0.083	0.02
DMN	S_N1	0.091	0.04
MNU	S_N1	0.099	0.02

TABLE III

INITIATION OF GGTase-POSITIVE FOCI BY METHYLATING CHEMICALS

Chemical Dose (mmol/kg)	Solvent[a]	N	Part. Hepatectomy[b]		GGTase-foci/cm²
			Before	After	
Diazald					
2.0	A	10	+	–	0.03 ± 0.03[c]
2.0	A	10	–	+	0.00 ± 0.00
DMH					
0.2	B	9	+	–	4.62 ± 0.92[d]
0.2	B	10	–	+	3.54 ± 0.70[d]
DMN					
0.1	B	9	+	–	1.49 ± 0.34[e]
0.3	B	10	–	+	4.22 ± 0.61[d]
DMS					
1.0	A	10	+	–	0.03 ± 0.03
1.0	A	10	–	+	0.00 ± 0.00
MMS					
0.3	B	10	+	–	0.10 ± 0.05
0.3	B	10	–	+	0.31 ± 0.11
MNNG					
1.0	C	9	+	–	1.14 ± 0.20[d]
1.0	C	10	–	+	0.90 ± 0.33
MNU					
0.3	D	10	+	–	1.38 ± 0.30[e]
0.3	D	9	–	+	0.16 ± 0.06
Solvent Controls					
a. DMSO	(A)	9	+	–	0.31 ± 0.11
b. Water	(B)	9	+	–	0.52 ± 0.23
c. Corn oil	(C)	8	+	–	0.18 ± 0.10
		8	–	+	0.18 ± 0.18

[a] Solvents: A, DMSO; B, water; C, corn oil; D, 1 mM sodium acetate (pH 5.6).
[b] Partial hepatectomy was performed either 20—22 h before or 2 weeks after the administration of the methylating chemical.
[c] Results are mean ± S.E.
[d] Different from control by the Student's t-test $P < 0.001$.
[e] Different from control by the Student's t-test $P < 0.05$.

decreased from 0.3 to 0.1 mmol/kg because of the high mortality observed at the higher dose. MNU and MNNG, which initiated a much smaller number of foci, were the only other methylating agents to demonstrate statistically significant initiation activity.

DISCUSSION

DNA alkylation and induction of GGTase-positive foci are two events that have been associated with the initiation of liver cancer [1—3]. In the case of methylating agents, initiation begins with the methylation of the DNA followed by the fixation of the alteration into the genome during DNA

transcription. The first observable evidence for initiation has been proposed to be the expression of altered foci of hepatocytes including GGTase-positive foci [1—3]. The neoplastic progression then continues to the formation of a tumor by a mechanism apparently susceptible to promotion.

Loveless [15] has proposed that O^6-alkylguanine is a mutagenic lesion that results in mispairing during DNA replication. Subsequent studies have also implicated the formation and persistence of O^6-alkylguanine with tumor production [6,17—21]. As a test of the correlation of O^6-alkylguanine to tumor production, we compared the ability of seven methylating agents to methylate the N-7 and O^6-positions in guanine and the 3-position in adenine with their ability to increase the incidence of GGTase-positive foci. 7-Methylguanine formation has been proposed to correlate with the total extent of DNA alkylation and O^6-methylguanine with cancer incidence. Only the methylating agents DMH, DMN, MNNG and MNU, which form O^6-methylguanine, initiated the formation of GGTase-positive foci. Craddock [22—24] has demonstrated that in partial hepatectomized rats a single dose DMN or MNNG (methylating agents that form O^6-methylguanine) induced liver tumors while MMS, which does not form O^6-methylguanine, did not induce tumors. Craddock [22—24] has also demonstrated that, although prior partial hepatectomy was required for the induction by methylating agents of liver tumor formation, prior partial hepatectomy did not alter the extent or type of DNA methylation. Qualitatively the ability to initiate GGTase-positive foci and tumors was associated with the formation of O^6-methylguanine.

The association of the induction of GGTase-positive foci with the formation of O^6-methylguanine indicates that the GGTase-positive focus originates from a cell containing a mutated genome. Mutagenic alteration of a specific site in the genome has been proposed to result in transformation of the cell. Clonal growth of the transformed cell and expression of the alteration results in the appearance of a focus of altered hepatocytes. Therefore, the association of GGTase-positive foci with O^6-methylguanine formation is indirect evidence that the foci are preneoplastic lesions.

The formation of O^6-methylguanine was not quantitatively related to the initiation of GGTase-positive foci. At the doses tested, DMH and DMN were equally potent in inducing GGTase-positive foci; however the extent of 7- and O^6-methylguanine and 3-methyladenine formation by DMN was approx. 20-fold greater than that found for DMH. Under the conditions of the present study, DMN and MNNG produced an equal amount of 7- and O^6-methylguanine although DMN was a much more potent inducer of GGTase-positive foci. Similarly although DMH was much more potent than MNU in initiating foci formation, the extent of MNU methylation at the 7- and O^6-position was 10-fold greater than that found for DMH. Farber and coworkers [25,26] have also shown that DMH, MNNG and MNU initiate GGTase-positive foci; however it was not possible to compare their relative potencies in these studies. It was also not possible to compare quantitatively the tumorigenic activity of DMN and MNNG that was demonstrated in partial hepatectomized

rats by Craddock [22—24]. More tumorigenicity studies, especially dose-response studies by these methylating agents in partially hepatectomized rats, are required to validate the observation that the extent of DNA methylation at either the 7- or O^6-position of guanine does not correlate with carcinogenic potency.

The lack of a quantitative correlation between O^6-methylguanine formation and formation of GGTase-positive foci could be the result of an effect of the methylating agents on the fixation and/or repair of the alteration in the DNA. Fixation of the alteration as a permanent change in the genome occurs during DNA transcription, so that, inhibition of DNA replication in the presence of continued DNA repair should decrease the amount of adduct remaining to cause mispairing during transcription. This would result in a decrease in the extent of initiation of cancer. Inhibition of DNA replication by some methylating agent has been demonstrated during the S-phase induced by partial hepatectomy [22—24] and in HeLa cells [27]. Unfortunately, the number of methylating agents tested in these systems is insufficient for an evaluation of the effect of their inhibition of DNA transcription on the relationship of DNA binding to carcinogenic potency. Another possible explanation for the lack of a quantitative relationship between O^6-methylguanine formation and GGTase-positive focus formation is that the differences in the toxicity of the methylating agents to the liver could result in differential loss by cell death of the more highly alkylated cells. In conclusion, the formation of O^6-methylguanine was demonstrated (1) to be associated with initiation of GGTase-positive foci and (2) for an as yet unknown reason not to correlate quantitatively with the formation of foci.

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